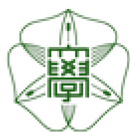
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1 Original Article

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3 Genetic and antigenic characterization of H5 and H7 avian influenza viruses isolated from 4 migratory waterfowl in Mongolia from 2017 to 2019

5

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14

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51 **Abstract:**

52 The circulation of highly pathogenic avian influenza viruses (HPAIVs) of various subtypes (e.g.,
53 H5N1, H5N6, H5N8, and H7N9) in poultry remains a global concern for animal and public health.
54 Migratory waterfowls play important roles in the transmission of these viruses across countries. To
55 monitor virus spread by wild birds, active surveillance for avian influenza in migratory waterfowl
56 was conducted in Mongolia from 2015 to 2019. In total, 5,000 fecal samples were collected from
57 lakesides in central Mongolia, and 167 influenza A viruses were isolated. Two H5N3, four H7N3, and
58 two H7N7 viruses were characterized in this study. The amino acid sequence at hemagglutinin (HA)
59 cleavage site of those isolates suggested low pathogenicity in chickens. Phylogenetic analysis was
60 revealed that all H5 and H7 viruses were closely related to recent H5 and H7 low pathogenic avian
61 influenza viruses (LPAIVs) isolated from wild birds in Asia and Europe. Antigenicity of H7Nx was
62 similar to those of typical non-pathogenic avian influenza viruses (AIVs). While HPAIVs or
63 A/Anhui/1/2013 (H7N9)-related LPAIVs were not detected in migratory waterfowl in Mongolia,
64 sporadic introductions of AIVs including H5 and H7 viruses into Mongolia through the wild bird
65 migration were identified. Thus, continued monitoring of H5 and H7 AIVs in both domestic and wild
66 birds is needed for the early detection of HPAIVs spread into the country.

67

68 **Keywords**: Avian influenza, Characterization, Migratory waterfowl, Mongolia, Surveillance

Introduction

Surveillance of avian influenza in wild birds has increased substantially worldwide in recent years because of the spread of H5 highly pathogenic avian influenza viruses (HPAIVs) among domestic poultry and wild birds in Asia, Europe, and Africa [1, 2]. Since the emergence of H5N1 HPAIVs in Asia [3], numerous global efforts have focused on elucidating the relative roles of wild bird and poultry movement in virus dissemination. To better understand the ecology of avian influenza viruses (AIVs) in wild birds, the data from wild bird surveillance studies are used to identify factors correlated with AIV detection in wild birds, such as reservoir species, bird health status, age, season, and location [4].

Each of the known subtype (H1–H16 and N1–N9) of influenza A virus (IAV) has been isolated from waterfowl, especially migratory wild ducks, that are infected with the viruses via waterborne transmission at their nesting lakes close to the Arctic Circle in Siberia, Alaska, and Canada during their breeding season in summer. These viruses replicate in columnar epithelial cells, forming crypts in the colon, and they are excreted in feces [5]. The infections of these AIVs do not cause illness in birds; however, current H5 HPAIVs that have been isolated in Asia, Europe, and Africa have caused death in several wild bird species [6].

Mongolia is located on three flyways of wild birds, namely the East Asian–Australasian, Central Asian, and East African–West Asian flyways, through which wild birds migrate from their northern territory in Siberia to the southern regions. In this context, intensive surveillance of AIVs in Mongolia has been conducted since autumn 1996 [7, 8]. Accordingly, the surveillance for migratory waterfowl in central Mongolia is essential for monitoring AIVs that were maintained in nesting lakes in Siberia and that spread southward with their migration, especially if recent H5 HPAIVs in Asia and H7N9 AIVs in China were brought to the north.

In the present study, to monitor AIVs among wild bird populations in Mongolia, fresh duck fecal samples were collected during autumn surveillance from 2015 to 2019. In total, 167 AIVs were

94 isolated from 5,000 fecal samples, and two H5N3, four H7N3, and two H7N7 viruses isolated in 2017
95 and 2019 were characterized. This study aimed to analyze H5 and H7 AIVs in Mongolia, genetically
96 and antigenically to clarify relations with recent H5 and H7 AIVs, especially HPAIVs and
97 A/Anhui/1/2013 (H7N9)-related low pathogenic avian influenza viruses (LPAIVs).
98
99 **Materials and Methods**
100 **Isolation and identification of viruses**
101 In each year, 1,000 duck fecal samples were collected in the central region of Mongolia,
102 including the Arkhangai Province (Ugii nuur, 47°76′N, 102°74′E; Doitiin tsagaan nuur, 47°37′N,
103 102°31′E; Duruu tsagaan nuur, 49°00′N, 101°12′E; Tsagaan nuur, 48°23′N, 102°35′E; Alagzegstei
104 nuur, 47°37′N, 102°32′E) and Bulgan Province (Khunt nuur 48°25′N, 102°34′E; Khunt rashaan nuur,
105 48°27′N, 102°32′E; Sharga nuur, 48°55′N, 101°56′E), annually from 2015 to 2019. Fecal samples
106 were stored at a temperature of less than 10°C and mixed with transport medium [minimal essential
107 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10,000 U/ml penicillin G, 10 mg/ml
108 streptomycin, 0.3 mg/ml gentamicin, and 0.5% bovine serum albumin] before virus isolation using
109 embryonated chicken eggs. At first, all samples were inoculated into the allantoic cavity of 10-day-
110 old chicken embryos and incubated for 48 h at 35°C. After incubation, the infectious allantoic fluid
111 was harvested, and the hemagglutination titer was determined using 0.5% chicken red blood cells.
112 For further characterization, the subtypes of influenza viruses were identified via hemagglutination
113 inhibition (HI) and neuraminidase inhibition (NI) tests using the reference antisera of AIVs [7].
114
115 **Sequencing and phylogenetic analysis**
116 For the genetic analysis, viral RNA was extracted from the allantoic fluid of infected chicken
117 embryos using TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA). Viral RNA was reverse-
118 transcribed using the Uni12 primer [9] and M-MLV reverse transcriptase (Life Technologies), then

119 the full-length hemagglutinin (HA) and other internal gene segments were amplified via polymerase
120 chain reaction using gene-specific primer sets [9]. Direct sequencing of gene segments was performed
121 using a BigDye Terminator version 3.1 Cycle Sequencing Kit (Life Technologies) and a 3500 Genetic
122 Analyzer (Life Technologies). For sequencing some isolates, next-generation sequencing was applied
123 as follows. MiSeq libraries were prepared using KAPA RNA Hyper Prep Kit (Illumina, Inc., San
124 Diego, CA, USA) and KAPA Dual-Indexed Adapter Kit (Roche, Basel, Switzerland). The prepared
125 MiSeq libraries were sequenced on a MiSeq by using MiSeq Reagent kit v3 (Illumina, Inc.) with 2 ×
126 300 bp paired-end read length. The sequencing data were analyzed using GENETYX Network
127 version 12 (Genetyx Co., Tokyo, Japan), GeneStudio (http://genestudio.com/), or CLC Genomics
128 Workbench 12 (Qiagen, Hilden, Germany). The gene sequences obtained in the present study have
129 been registered at GenBank (Supplemental Table 1).

130 Phylogenetic analysis of H5 or H7 HA gene was performed by the maximum likelihood (ML)
131 method using the Tamura-Nei model and bootstrap analysis (n = 1000) using MEGA7.0 software
132 with default parameters [10]. The sequence data from two H5 HA and six H7 HA genes were
133 compared with the reference sequences obtained from public databases, respectively. For the
134 reference sequences, the nucleotide sequences of classical and recent H5 and H7 viruses were
135 downloaded from GenBank/EMBL/DDBJ and Global Initiative on Sharing All Influenza Data
136 (GISAID).

137

138 **Antigenic analysis**

139 The antigenic properties of representative H7Nx isolates were determined via the cross-HI test
140 using chicken polyclonal antisera. Chicken polyclonal antisera against representative influenza virus
141 strains were prepared, and HI tests were performed as previously described [11]. To visualize the
142 antigenic character of the H7 viruses, the antigenic map was built using the web-based software
143 (https://acmacs-web.antigenic-cartography.org/), and the test result containing cross-HI titers were

144 uploaded to obtain x/y coordinates of each antiserum and antigen.

145

146 **Results**

147 **Isolation of IAVs from the fecal samples of migratory waterfowl**

148 The surveillance was targeted migratory waterfowl in four main lakes located in the Arkhangai
149 and Bulgan Province of Mongolia. In total, 167 influenza viruses were isolated from 5,000 fecal
150 samples of migratory waterfowl (Table 1 and Supplemental Table 1); respectively, 40, 10, 21, 73,
151 and 23 AIVs were isolated each autumn from 2015 to 2019. The isolation rate of AIVs for each
152 autumn over five years was 1.0%–7.3%, and the highest number of isolated viruses was observed in
153 the Arkhangai Province. Among each sampling site, Doitiin Tsagaan nuur in the Arkhangai Province
154 and Khunt nuur in the Bulgan Province were found to be the highest percentage of isolated virus
155 during the overall surveillance time. The majority of HA subtypes of all isolates was H3 and H4.
156 In the present study, further characterization was conducted using two H5N3 and six H7Nx
157 isolates. The two H5N3 isolates were A/duck/Mongolia/419/2019 and A/duck/Mongolia/926/2019
158 from the Arkhangai and Bulgan Provinces. Four H7N3 isolates were A/duck/Mongolia/652/2017,
159 A/duck/Mongolia/751/2017, A/duck/Mongolia/782/2017, and A/duck/Mongolia/786/2017 collected
160 in Bulgan Province. Two H7N7 isolates, namely A/duck/Mongolia/1/2019 and
161 A/duck/Mongolia/6/2019, were collected in Arkhangai Province.

162

163 **Genetic and phylogenetic analysis of H5 and H7 subtype viruses**

164 The full-length HA gene sequences of the H5 and H7 isolates were characterized. The deduced
165 amino acid sequences of the HA cleavage site of the two H5N3 isolates were PQRETR/GLF and
166 PQREIR/GLF, indicating that they were LPAIVs. The HA receptor-binding site of the H5N3 isolates
167 was analyzed, and residues at positions 226 and 228 were identified as Q and G, respectively,
168 suggesting avian-type receptor specificity. None of the H5N3 subtype viruses contained amino acid

169 substitutions E627K in PB2, which is the molecular marker for mammalian adaptation of AIVs and
170 indicates increasing viral pathogenicity to mammals. Furthermore, the HA cleavage site of the four
171 H7N3 and two H7N7 isolates were analyzed, and the determined sequence was PELPKGR/GLF and
172 PEIPKGR/GLF, respectively, suggesting that the viruses were LPAIVs. The HA receptor-binding
173 site of H7Nx isolates was also analyzed, and HA proteins of isolates had residues of 226Q and 228G,
174 indicating avian-type receptor specificity. Similarly, H7Nx LPAIVs did not involve a mutation for
175 potential mammalian adaptation in PB2 protein. Taken together, these data indicate that all H5 and
176 H7 isolates were LPAIVs without mammalian adaptation markers.

177 The HA gene of these isolates was phylogenetically analyzed by the ML method along with
178 reference strains of HPAIVs and LPAIVs (Figs. 1 and 2). In this phylogenetic analysis, H5 HA genes
179 were divided into two lineages: Eurasian and North American. The Eurasian lineage was clustered
180 into two sub-lineages: Gs/Gd-like and non-Gs/Gd [12]. All H5N3 isolates from migratory waterfowl
181 in Mongolia in 2019 were belonged to the non-Gs/Gd sub-lineage that clustered with H5N3 subtype
182 viruses recently reported in Asia and were distinct from Gs/Gd-like viruses. For other gene segments,
183 each segment was classified together with multivariable subtype viruses circulating among wild birds
184 (Supplemental Figs. S1–S5, S7, S8). Moreover, the H7 HA genes were phylogenetically divided into
185 several lineages: Eurasian, Australian, Historical Europe, and North American. Eurasian lineage was
186 further clustered into three sub-lineages: European–Asian, Far Eastern, and Chinese H7N9 [7]. All
187 H7Nx isolates isolated in Mongolia in 2017 and 2019 were belonged to the European–Asian sub-
188 lineage. Reference strains of Chinese H7N9 viruses were classified together with the Far Eastern sub-
189 lineage; therefore, H7N3 and H7N7 isolates of migratory waterfowls had a different ancestor than
190 that of Chinese H7N9 viruses. Other gene segments of H7N3 and H7N7 viruses were also classified
191 with typical LPAIVs circulating in wild birds (Supplemental Figs. S1–S8) [13].

192 In phylogenetic analysis, the N3 and N7 neuraminidase (NA) genes were classified with
193 Eurasian lineage viruses and had a genetic relationship with other variable N3 and N7 subtype viruses

194 including AIVs isolated in Mongolia in 2010 and 2015. All other internal genes of H7N3 and H7N7
195 were phylogenetically closely related to recent H7 subtype viruses isolated from wild birds in Asia
196 and Europe.
197
198 **Antigenic analysis of H7Nx subtype viruses**
199 Representative strains of H7 viruses from Mongolia and the panel of H7 viruses belonging to
200 Eurasian and North American lineage were antigenically analyzed by the cross-HI test using their
201 corresponding antisera (Table 2). HI titers of each antiserum against A/duck/Mongolia/652/2017
202 (H7N3) and A/duck/Mongolia/1/2019 (H7N7) were closely identical to those against previously
203 isolated H7N2 viruses classified in the European–Asian sub-lineage [7]. The antigenic cartography
204 was built from the cross-HI test which revealed that present H7N3 and H7N7 strains were
205 antigenically closely related to recent H7 viruses isolated from wild birds. These belonged to the
206 major antigenic group and were distinct from H7 HPAIVs (Supplemental Fig. S9).
207
208 **Discussion**
209 H5 or H7 HPAIVs as well as LPAIVs are a global threat to livestock production, distribution
210 systems, and human health. However, sustained, comprehensive, coordinated global efforts to
211 monitor the continually changing genetic diversity of AIVs circulating in nature remain insufficient
212 to predict the emergence of novel HPAIVs and human infections of AIVs. Wild aquatic birds have
213 played a role in the evolution of H5 HPAIVs by reassortment with LPAIVs. These factors have
214 contributed to the spread of these viruses to parts of Asia, Europe, Africa, and North America after an
215 outbreak in wild birds at Qinghai Lake in China in 2005 [14]. Circulations of H7 LPAIVs in wild
216 birds and poultry have been reported throughout the world [7, 15-19, 13, 20]. In addition, H7N9
217 HPAIVs and LPAIVs are at risk to spread by wild bird and human-caused movements [21, 22]. In
218 our surveillance conducted over the past five years, targeting migratory waterfowl in four different

219 lakes in the central region of Mongolia, 167 viruses were isolated. No HPAIVs were isolated from
220 the fecal samples of migratory waterfowl in the present study, indicating that the circulation of
221 HPAIVs in the northern nesting lake is still limited. However, these do not fully represent the country
222 state in terms of the infection of IAV in wild birds.

223 Two AIVs are currently pandemic threats; H5 HPAIVs have spilled over repeatedly to humans
224 since their first identification in 1997, and H7N9 AIVs, initially detected in March 2013, have caused
225 serious human infections across China [23]. In terms of H7N9 virus infection in humans, a total of
226 1,568 laboratory-confirmed human cases have been reported since early 2013 [24]. A novel H7N9
227 HPAIV variant possessing multiple basic amino acids at the cleavage site of the HA protein was first
228 reported in two cases of human infection in January 2017 [25]. H5N3 LPAIVs were detected from
229 migratory waterfowl in Mongolia in 2019, and HA gene and other gene segments of those H5 isolates
230 were phylogenetically classified in the Eurasian lineage, sharing identical genetic characteristics with
231 recent H5 LPAIVs isolated from wild birds in Asia. Phylogenetic analysis of H7N3 and H7N7 isolates
232 was revealed a close relation to H7 LPAIVs belonging to the European-Asian sub-lineage, but no
233 relation to Chinese H7N9 viruses.

234 Antigenicity monitoring is important for diagnosis and control measures for HPAIVs and
235 LPAIVs. In the present study, representative H7 viruses were analyzed for antigenic characterization.
236 Kim et al. reported the antigenic difference of viruses in Eurasian group I (referred to as the Far
237 Eastern sub-lineage in this study) and group II (Eurasian–Asian sub-lineage) [15]. Our antigenic
238 analyses revealed that the antigenicity of H7 viruses isolated from migratory waterfowl was similar
239 to each other regardless of genetic diversity, and antigenic diversity may emerge after the adaptation
240 of H7 viruses to poultry, same as has been reported for North American H7 viruses [19].

241 Because of the recent increased numbers of zoonotic infections in poultry and human infections
242 in China, influenza A virus (H7N9) has remained a public health threat. It is possible that wild birds
243 infected with Chinese H7N9 viruses transmit them to surrounding countries, as occurs with H5

244 HPAIVs. Continued circulation of H7 influenza viruses among poultry and wild birds risks
245 widespread dissemination of these viruses. Accordingly, it is important that H5 and H7 viruses are
246 continually monitored among wild waterfowl.
247
248 **Statement of author contributions:**
249 **:** AU and EB performed genetic analyses and antigenic analyses. AU and EB prepared this manuscript.
250 AU, EB, TH, KB, TA, JT, TU, KhB, KM, TH, TK, MS, YT, ST, MI, KO, TS, NK, YK, JM, MI, and
251 AT conducted sampling of feces from migratory wild birds in Mongolia and performed virus isolation
252 and subtyping. MO, KM, AT, HK, DB, and YS provided laboratory management support and
253 manuscript editing.
254
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262 and Animal Husbandry of Mongolia in the Japan International Cooperation Agency (JICA).
263
264 **Figure legends**
265 **Fig. 1.** Phylogenetic tree for H5 HA genes of AIVs. Full-length sequences of HA genes of two H5
266 subtype viruses were analyzed by the ML method along with those of reference strains using
267 MEGA7.0 software. The horizontal distances are proportional to the minimum number of nucleotide
268 differences required to join nodes and sequences. Digits at the nodes indicate the probability of

269 confidence levels in a bootstrap analysis with 1000 replications. The numbers below or above the
270 node indicate bootstrap values ≥60%. The viruses isolated in this study are highlighted in gray. The
271 previous isolates in our surveillance are indicated with black triangle symbol. HPAIVs are indicated
272 in bold.
273
274 **Fig. 2.** Phylogenetic tree for the H7 HA genes of IAVs. Full-length sequences of HA genes of six H7
275 subtype viruses were analyzed by the ML method along with those of reference strains using
276 MEGA7.0 software. The horizontal distances are proportional to the minimum number of nucleotide
277 differences required to join nodes and sequences. Digits at the nodes indicate the probability of
278 confidence levels in a bootstrap analysis with 1000 replications. The numbers below or above the
279 node indicate bootstrap values ≥60%. The viruses isolated in this study are highlighted in gray. The
280 previous isolates in our surveillance are indicated with black triangle symbol. Highly pathogenic IAVs
281 are indicated in bold and Chinese H7N9 viruses are underlined.
282
283 **Supplemental Figs. S1–S8.** Phylogenetic tree for the PB2 (S1), PB1 (S2), PA (S3), NP (S4), N3 NA
284 and N7 NA (S5), M (S6), and NS (S7) genes of IAVs. Full-length sequences of other gene segments
285 of H5 and H7 subtype viruses isolated in the present study were analyzed by the ML method along
286 with those of reference strains using MEGA7.0 software. Horizontal distances are proportional to the
287 minimum number of nucleotide differences required to join nodes and sequences. Digits at the nodes
288 indicate the probability of confidence levels in a bootstrap analysis with 1000 replications. The
289 numbers below or above the node indicate bootstrap values ≥60%. The viruses isolated in this study
290 are highlighted in gray. The previous isolates in our surveillance are indicated with black triangle
291 symbol. Highly pathogenic IAVs are indicated in bold and Chinese H7N9 viruses are underlined.
292

293 **Supplemental Fig. S9.** Antigenic cartography for H7N3 and H7N7 viruses isolated in Mongolia. The
294 cartography was constructed (https://acmacs-web.antigenic-cartography.org/) based on the HI data in
295 Table 2. Each gridline (horizontal and vertical) represents antigenic distance as a two-fold difference
296 in HI titer. Each antiserum was linked to a dashed line with homologues antigen. The antisera and
297 antigen are indicated by the square and the circle symbol, respectively. The antigenic group is
298 highlighted with blue color. The viruses isolated in this study are highlighted with yellow color.
299 LPAIVs and HPAIVs are written in black and red letters, respectively.
300
301 **Conflict of Interest:** The authors declare no conflict of interest.
302 **Ethical approval:** This article does not contain any studies with human participants or animals
303 performed by any of the authors
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318 **References**

319 1. Machalaba CC, Elwood SE, Forcella S, Smith KM, Hamilton K, Jebara KB, Swayne DE, Webby RJ,
320 Mumford E, Mazet JA, Gaidet N, Daszak P, Karesh WB (2015) Global avian influenza surveillance in
321 wild birds: a strategy to capture viral diversity. Emerg Infect Dis 21:e1-7.
322 http://dx.doi.org/10.3201/eid2104.141415

323 2. OIE website (2019) Update on highly pathogenic avian influenza in animals (Types H5 and H7).
324 http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/.

325 3. Xu X, Subbarao, Cox NJ, Guo Y (1999) Genetic characterization of the pathogenic influenza
326 A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1
327 viruses from the 1997 outbreaks in Hong Kong. Virology 261:15-19.
328 https://doi.org/10.1006/viro.1999.9820

329 4. Rose K, Newman S, Uhart M, Lubroth J (2006) Wild bird highly pathogenic avian influenza
330 surveillance: Sample collection from healthy, sick and dead birds. FAO Animal Production and Health
331 Manual No.4. http://www.fao.org/3/a-a0960e.pdf. Assessed 20 May 2019

332 5. Kida H, Yanagawa R, Matsuoka Y (1980) Duck influenza lacking evidence of disease signs and
333 immune response. Infect and Immun 30:547-553

334 6. Lycett SJ, Bodewes R, Pohlmann A, Banks J, Banyai K, Boni MF, Bouwstra R, Breed AC, Brown IH,
335 Chen HL, Dan A, DeLiberto TJ, Diep N, Gilbert M, Hill S, Ip HS, Ke CW, Kida H, Killian ML, Koopmans
336 MP, Kwon JH, Lee DH, Lee YJ, Lu L, Monne I, Pasick J, Pybus OG, Rambaut A, Robinson TP, Sakoda Y,
337 Zohari S, Song CS, Swayne DE, Torchetti MK, Tsai HJ, Fouchier RAM, Beer M, Woolhouse M, Kuiken T
338 (2016) Role for migratory wild birds in the global spread of avian influenza H5N8. Science 354:213-
339 217. https://doi.org/10.1126/science.aaf8852

340 7. Hiono T, Ohkawara A, Ogasawara K, Okamatsu M, Tamura T, Chu DH, Suzuki M, Kuribayashi S,
341 Shichinohe S, Takada A, Ogawa H, Yoshida R, Miyamoto H, Nao N, Furuyama W, Maruyama J, Eguchi
342 N, Ulziibat G, Enkhbold B, Shatar M, Jargalsaikhan T, Byambadorj S, Damdinjav B, Sakoda Y, Kida H
343 (2015) Genetic and antigenic characterization of H5 and H7 influenza viruses isolated from migratory
344 water birds in Hokkaido, Japan and Mongolia from 2010 to 2014. Virus Genes 51:57-68.
345 https://doi.org/10.1007/s11262-015-1214-9

346 8. Sakoda Y, Sugar S, Batchluun D, Erdene-Ochir TO, Okamatsu M, Isoda N, Soda K, Takakuwa H, Tsuda
347 Y, Yamamoto N, Kishida N, Matsuno K, Nakayama E, Kajihara M, Yokoyama A, Takada A,
348 Sodnomdarjaa R, Kida H (2010) Characterization of H5N1 highly pathogenic avian influenza virus
349 strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to
350 their northern territory. Virology 406:88-94. https://doi.org/10.1016/j.virol.2010.07.007

351 9. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length
352 amplification of all influenza A viruses. Arch Virol 146:2275-2289.
353 https://doi.org/10.1007/s007050170002

354 10. Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version

355 7.0 for Bigger Datasets. Mol Biol Evol 33:1870-1874. https://doi.org/10.1093/molbev/msw054
356 11. Sakabe S, Sakoda Y, Haraguchi Y, Isoda N, Soda K, Takakuwa H, Saijo K, Sawata A, Kume K,
357 Hagiwara J, Tuchiya K, Lin Z, Sakamoto R, Imamura T, Sasaki T, Kokumai N, Kawaoka Y, Kida H (2008)
358 A vaccine prepared from a non-pathogenic H7N7 virus isolated from natural reservoir conferred
359 protective immunity against the challenge with lethal dose of highly pathogenic avian influenza virus
360 in chickens. Vaccine 26:2127-2134. https://doi.org/10.1016/j.vaccine.2008.02.001
361 12. Nguyen LT, Firestone SM, Stevenson MA, Young ND, Sims LD, Chu DH, Nguyen TN, Nguyen LV, Le
362 TT, Nguyen HV, Nguyen HN, Tien TN, Nguyen TD, Tran BN, Matsuno K, Okamatsu M, Kida H, Sakoda
363 Y (2019) A systematic study towards evolutionary and epidemiological dynamics of currently
364 predominant H5 highly pathogenic avian influenza viruses in Vietnam. Sci Rep 9:1-13.
365 https://doi.org/10.1038/s41598-019-42638-4
366 13. Le KT, Okamatsu M, Nguyen LT, Matsuno K, Chu DH, Tien TN, Le TT, Kida H, Sakoda Y (2020)
367 Genetic and antigenic characterization of the first H7N7 low pathogenic avian influenza viruses
368 isolated in Vietnam. Infect Genet Evol 78:1-8. https://doi.org/10.1016/j.meegid.2019.104117
369 14. Liu J, Xiao H, Lei F, Zhu Q, Qin K, Zhang XW, Zhang XL, Zhao D, Wang G, Feng Y, Ma J, Liu W, Wang
370 J, Gao GF (2005) Highly pathogenic H5N1 influenza virus infection in migratory birds. Science
371 309:1206, 1-2. https://doi.org/10.1126/science.1115273
372 15. Kim YI, Kim SW, Si YJ, Kwon HI, Park SJ, Kim EH, Kim SM, Lee IW, Song MS, Choi YK (2016) Genetic
373 diversity and pathogenic potential of low pathogenic H7 avian influenza viruses isolated from wild
374 migratory birds in Korea. Infec Genet Evol 45:268-284.
375 https://doi.org/10.1016/j.meegid.2016.09.005
376 16. Kim HR, Park CK, Lee YJ, Oem JK, Kang HM, Choi JG, Lee OS, Bae YC (2012) Low pathogenic H7
377 subtype avian influenza viruses isolated from domestic ducks in South Korea and the close
378 association with isolates of wild birds. J Gen Virol 93:1278-1287.
379 https://doi.org/10.1099/vir.0.041269-0
380 17. Liu H, Xiong C, Chen J, Chen G, Zhang J, Li Y, Xiong Y, Wang R, Cao Y, Chen Q, Liu D, Wang H, Chen
381 J (2018) Two genetically diverse H7N7 avian influenza viruses isolated from migratory birds in central
382 China. Emerg Microbes Infect 7:62, 1-12. https://doi.org/10.1038/s41426-018-0064-7
383 18. Yao Y, Xu CL, Shi JH, Zhu Y, Li YF, Bai T, Li FC, Cai T, Yuan F, Chen T, Yang H, Li WC, Zhang HJ, Zhang
384 H, Shu YL (2015) Phylogenetic and Molecular Analysis of an H7N7 Avian Influenza Virus Isolated in
385 East Dongting Lake in 2012. Biomed Environ Sci 28:518-526. https://doi.org/10.3967/bes2015.074
386 19. Xu Y, Bailey E, Spackman E, Li T, Wang H, Long LP, Baroch JA, Cunningham FL, Lin X, Jarman RG,
387 DeLiberto TJ, Wan XF (2016) Limited Antigenic Diversity in Contemporary H7 Avian-Origin Influenza
388 A Viruses from North America. Sci Rep 6:20688,1-17. https://doi.org/10.1038/srep20688
389 20. Suttie A, Yann S, Y P, Tum S, Deng YM, Hul V, Horm VS, Barr I, Greenhill A, Horwood PF, Osbjer K,
390 Karlsson EA, Dussart P (2018) Detection of Low Pathogenicity Influenza A(H7N3) Virus during Duck
391 Mortality Event, Cambodia, 2017. Emerg Infect Dis 24:1103-1107.
392 https://doi.org/10.3201/eid2406.172099

393 21. Shibata A, Okamatsu M, Sumiyoshi R, Matsuno K, Wang ZJ, Kida H, Osaka H, Sakoda Y (2018)
394 Repeated detection of H7N9 avian influenza viruses in raw poultry meat illegally brought to Japan
395 by international flight passengers. Virology 524:10-17. https://doi.org/10.1016/j.virol.2018.08.001
396 22. Shibata A, Harada R, Okamatsu M, Matsuno K, Arita T, Suzuki Y, Shirakura M, Odagiri T, Takemae
397 N, Uchida Y, Saito T, Sakoda Y, Osaka H (2019) Characterization of a novel reassortant H7N3 highly
398 pathogenic avian influenza virus isolated from a poultry meat product taken on a passenger flight to
399 Japan. J Vet Med Sci 81:444-448. https://doi.org/10.1292/jvms.18-0628
400 23. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, Xu X, Lu H, Zhu W, Gao Z,
401 Xiang N, Shen Y, He Z, Gu Y, Zhang Z, Yang Y, Zhao X, Zhou L, Li X, Zou S, Zhang Y, Li X, Yang L, Guo J,
402 Dong J, Li Q, Dong L, Zhu Y, Bai T, Wang S, Hao P, Yang W, Zhang Y, Han J, Yu H, Li D, Gao GF, Wu G,
403 Wang Y, Yuan Z, Shu Y (2013) Human infection with a novel avian-origin influenza A (H7N9) virus. N
404 Engl J Med 368:1888-1897. https://doi.org/10.1056/NEJMoa1304459
405 24. World Health Organization (2019) Human infection with avian infleuzna A(H7N9) virus.
406 https://www.who.int/csr/don/05-september-2018-ah7n9-china/en/. Accessed May 23 2019
407 25. Ke C, Mok CKP, Zhu W, Zhou H, He J, Guan W, Wu J, Song W, Wang D, Liu J, Lin Q, Chu DKW, Yang
408 L, Zhong N, Yang Z, Shu Y, Peiris JSM (2017) Human Infection with Highly Pathogenic Avian Influenza
409 A(H7N9) Virus, China. Emerg Infect Dis 23:1332-1340. https://doi.org/10.3201/eid2308.170600
410

Table 1. Influenza viruses isolated from migratory waterfowls in Mongolia during the surveillance in autumn between 2015 and 2019

Location	Subtypes of influenza virus				
	2015 (40/ 1,000 samples)	2016 (10/ 1,000 samples)	2017 (21/ 1,000 samples)	2018 (73/ 1,000 samples)	2019 (23/ 1,000 samples)
Arkhangai province	H1N1 (1) H1N2 (1) H3N8 (15) H4N6 (3) H6N2 (1) H10N2 (2) H10N3 (2)	H3N8 (1) H4N6 (6)	H2N2 (1) H3N8 (4) H4N6 (9) H10N9 (1)	H2N3 (8) H2N4 (3) H3N6 (3) H3N8 (24) H4N1 (1) H4N6 (17) H12N5 (2)	H3N6 (2) H3N8 (2) H4N2 (3) H4N6 (7) **H5N3 (1)** **H7N7 (2)**
Bulgan province	H1N1 (1) H2N3 (2) H3N6 (1) H3N8 (4) H4N6 (2) H10N3 (1) H10N7 (4)	H3N8 (1) H4N6 (2)	H3N8 (2) **H7N3 (4)**	H3N1 (1) H3N8 (10) H12N5 (4)	H3N7 (1) H3N8 (2) H4N2 (1) H4N6 (1) **H5N3 (1)**

H5 and H7 viruses are shown in bold.

The number of isolates of each antigenic subtype is shown in parenthesis.

Table 2. Cross HI test of H7 influenza viruses with polyclonal antibodies

Lineage	Viruses	Subtype	HI titers of the antiserum						
			Dk/Hok/ W19/13	Ty/Italy/4 580/99	Dk/Hok/V ac-2/04	Anhui/1/ 13	Ck/NSW/ 327/97	Dk/TW/Y a103/93	Sl/Mass/1 /80
Eurasian									
European–Asian	**A/duck/Mongolia/652/2017**	H7N3	10,240	1,280	5,120	1,280	5,120	640	320
	A/duck/Mongolia/1/2019	H7N7	10,240	1,280	5,120	1,280	5,120	1,280	1,280
	A/duck/Hokkaido/W19/2013	H7N2	2,560	1,280	5,120	1,280	2,560	320	320
	A/turkey/Italy/4580/1999	H7N1	320	1,280	160	80	320	80	80
Far-Eastern	A/duck/Hokkaido/Vac-2/2004	H7N7	20,480	2,560	20,480	2,560	10,240	1,280	1,280
Chinese H7N9	A/Anhui/1/2013	H7N9	5,120	1,280	5,120	2,560	5,120	320	320
Australian	A/chicken/New South Wales/327/1997	H7N4	5,120	1,280	1,280	640	5,120	320	320
Historical Europe	A/duck/Taiwan/Ya103/1993	H7N7	320	80	160	160	160	2,560	40
North American	A/seal/Massachusetts/1/1980	H7N7	10,240	2,560	20,480	2,560	10,240	320	2,560

Dk: duck, Ty: turkey, Ck: chicken, Sl: seal, Hok: Hokkaido, NSW: New South Wales, TW: Taiwan, Mass: Massachusetts.
Viruses isolated in this study are shown in bold.
Homologous titers are indicated with underline.

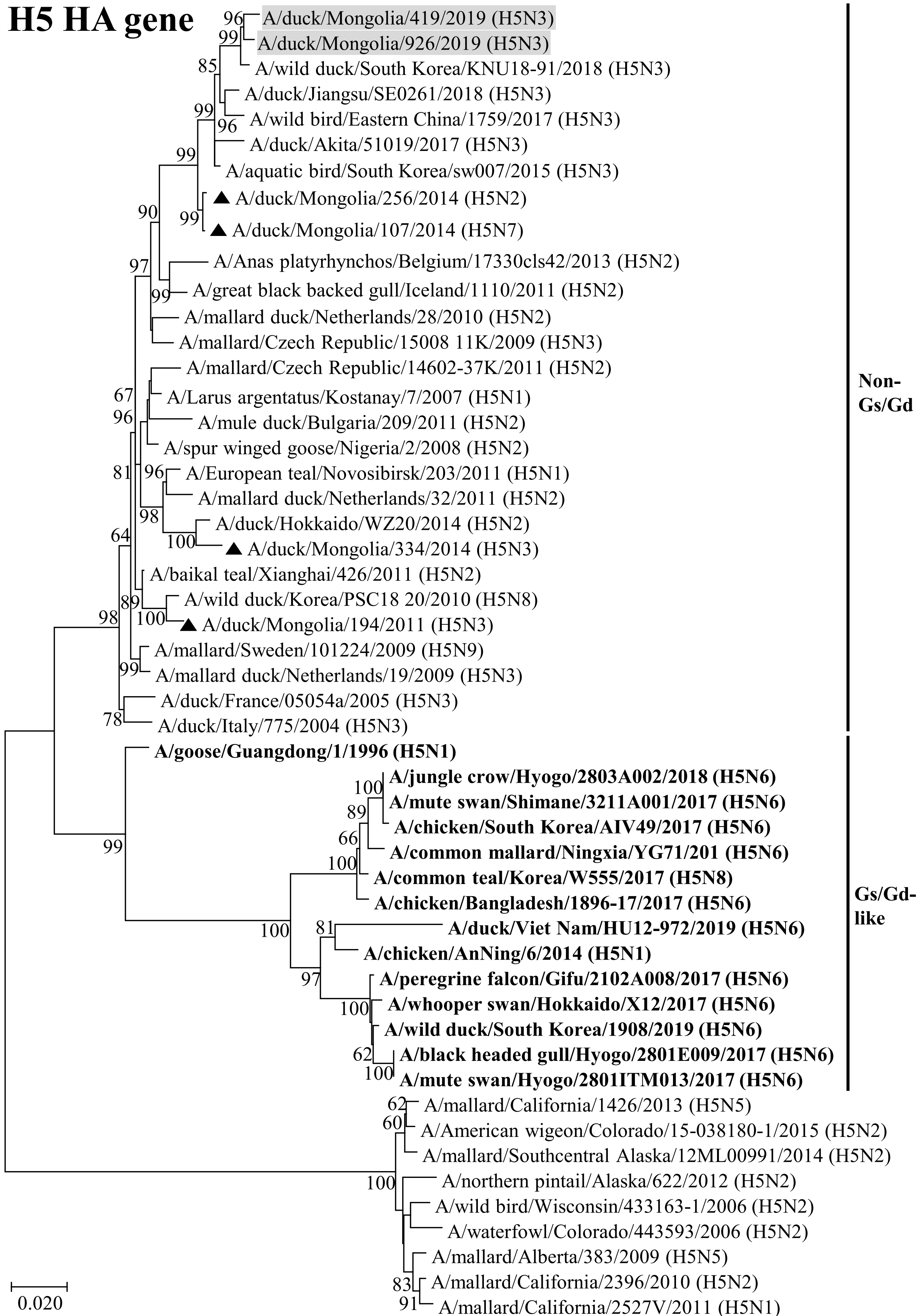

Fig. 1. Ulaankhuu *et al.*

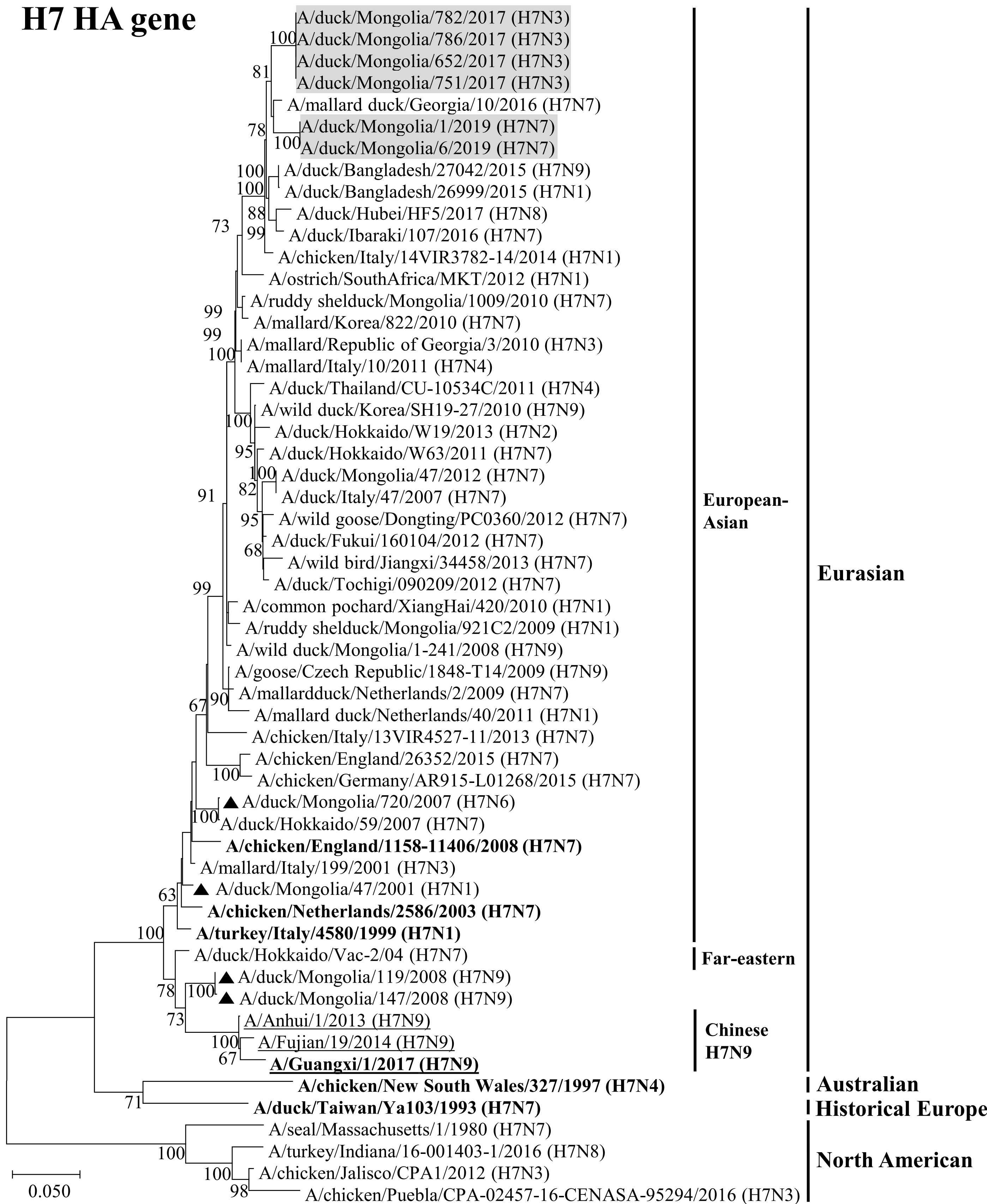

Fig. 2. Ulaankhuu *et al.*